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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 31/00, 31/725, 39/395

A1

(11) International Publication Number: WO 94/16684

(43) International Publication Date: 4 August 1994 (04.08.94)

(21) International Application Number: PCT/US94/00516

(22) International Filing Date: 26 January 1994 (26.01.94)

(30) Priority Data:
08/009,595 26 January 1993 (26.01.93) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: INHIBITION OF βARK AND β-ARRESTIN

(57) Abstract

β-adrenergic receptor kinase (βARK) and β-arrestin function in the homologous or agonist-activated desensitization of G-protein coupled receptors. Both βARK-2 and β-arrestin-2 isoforms are highly enriched in and localized to the cilia and dendritic knobs of the olfactory receptor neurons where the initial events of olfactory signal transduction occur as well as being localized to spermatids. Administration of neutralizing antibodies to βARK-2 and β-arrestin-2 enhances the response to odorants and attenuates desensitization.

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INHIBITION OF βARK AND β-ARRESTIN

TECHNICAL FIELD OF THE INVENTION

The invention relates to the field of cellular receptors for sensory signal transduction. In particular, it relates to inhibition of cellular receptors involved in olfaction and sperm chemotaxis.

BACKGROUND OF THE INVENTION

The primary events of olfactory signal transduction occur in cilia of olfactory receptor neurons (ORN) and proceed through the generation of the intracellular second messengers cAMP and inositol 1,4,5-trisphosphate ($InsP_3$) (U. Pace et al., Nature 316, 225 (1985); P.B. Sklar, et al., J. Biol. Chem., 261, 15538 (1986); H. Breer and I. Boekhoff, Nature 345, 65 (1990); G.V. Ronnett et al., Proc. Natl. Acad. Sci., USA 88, 2366 (1991), D. Restrepo et al., Science 249, 1166 (1990)). Isoforms of several components of these transduction pathways are highly enriched in olfactory neuronal cilia and include a Type III adenylyl cyclase (H.A. Bakalyar et al., Science 250, 1403 (1989)), a cyclic nucleotide-gated nonspecific cation channel (R.S. Dhallan et al., Nature 347, 184 (1990); J. Ludwig et al., FEBS Lett. 270, 24 (1990); S. Firestein et al., J. Neurosci., 11, 3565 (1991); T. Nakamura and

G.H. Gold, Nature 325, 442 (1987)), and an alpha subunit of a stimulatory G protein, G_{olf} (D.T. Jones et al., Science 244, 790 (1989).). The membrane receptors presumed to transduce the odorant stimulus are members of a large G protein-linked, seven transmembrane domain receptor gene family (L. Buck and R. Axel, Cell 65, 175 (1991)) and are similarly localized in the olfactory neuron (A.C. Cunningham et al., Neurosci. Abstr, 17, 75.4 (1991)). A large body of evidence (R.S. Dhallan et al., supra; J. Ludwig, et al. supra; S. Firestein, et al. supra; T. Nakamura, et al. supra) indicates that the odorant-induced elevation in intracellular cAMP initiates electrophysiological responses involved in the generator potential by directly activating a nonspecific cation channel located in the plasma membrane of the sensory cilia. As with other responses mediated by G protein-coupled receptors, odorant stimulated signals display rapid attenuation even in the face of a constant stimulus, a phenomenon termed desensitization. Although the initial events of olfactory signal transduction have been extensively explored, the mechanisms involved in desensitization to odorants are less clear.

Desensitization of signal transduction may occur through a variety of processes, including receptor internalization, or receptor uncoupling mediated by receptor phosphorylation (D.R. Sibley et al., Cell, 48, 913 (1987); W.L. Klein et al., FASEB J, 3, 2132 (1980); R.L. Huganir et al., Neuron 5, 555 (1990); J.L. Benovic et al., Ann. Rev. Cell Biol., 4, 405 (1988); W.P. Hausdorff et al., FASEB J., 4, 2881 (1990)). Utilizing the β_2-adrenergic receptor as a model system, the functional consequences of G protein-coupled receptor phosphorylation have been

extensively characterized (J.L. Benovic et al., Ann. Rev. Cell Biol., 4, 405 (1988); W.P. Hausdorff et al., FASEB J., 4, 2881 (1990)). Homologous or agonist-specific desensitization of the β_2-adrenergic receptor occurs through agonist activated receptor phosphorylation that is catalyzed by a specific receptor kinase called β-adrenergic receptor kinase (βARK) (J.L. Benovic et al., Science 246, 235 (1989)). Further quenching of signal transduction requires the binding of a protein called β-arrestin to phosphorylated receptors (M.J. Lohse et al., Science 248, 1547 (1990); J.L. Benovic et al., Proc. Natl. Acad. Sci., USA 84, 8879 (1987)). To date three receptor kinases have been identified: rhodopsin kinase (W. Lorenz et al., Proc. Natl. Acad. Sci., USA 88, 8715 (1991)), βark-1 (M.J. Lohse et al., Science 248, 1547 (1990); J.L. Benovic et al., Proc. Natl. Acad. Sci., USA 84, 8879 (1987)), and βARK-2 (J.L. Benovic et al., J. Biol. Chem. 266, 14939 (1991)). Additionally three different arrestins are known, arrestin (T. Shinohara et al., Proc. Natl. Acad. Sci., USA 84, 6974 (1987)), β-arrestin-1 (M.J. Lohse et al., Science 248, 1547 (1990); J.L. Benovic et al., Proc. Natl. Acad. Sci., USA 84, 8879 (1987)), and β-arrestin-2 (Attramadal et al., J. Biol. Chem., 267, 17882 (1992)).

There is a need in the art for methods of enhancing the perception of odorants and for methods of inhibiting the desensitization to odorants.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for inhibiting the desensitization to odorants or for enhancing the perception of odorants in a subject.

It is another object of the invention to provide a kit for diagnosing a cause of anosmia.

It is still another object of the invention to provide a process for testing odorant perception in a subject who cannot smell an odorant.

It is an object of the invention to provide methods for affecting the fertility of mammals.

It is yet another object of the invention to provide a composition for affecting the fertility of mammals.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method for inhibiting the desensitization to odorants or for enhancing the perception of odorants in a subject is provided. The method comprises: administering a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin, to the olfactory epithelium of the subject in an amount sufficient to inhibit the desensitization or to enhance the perception of an odorant.

In another embodiment of the invention a kit for diagnosing a cause of anosmia is provided. The kit comprises: one or more odorants; a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin; a means for indicating perception of an odorant.

In still another embodiment of the invention a process for testing odorant perception in a subject who cannot smell an odorant is provided. The process

comprises the steps of: exposing the subject to an odorant at a concentration at which the subject cannot perceive the odorant; administering to the subject a molecule which inhibits an olfactory protein selected from the group consisting of βARK and β-arrestin; determining whether administration of said molecule allows the subject to perceive the odorant.

In yet another embodiment of the invention a method for affecting fertility of mammals is provided. The method comprises: applying to mammalian sperm a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin in an amount sufficient to attenuate the desensitization or enhance the response of said sperm to odorants; and artificially inseminating a female mammal with said treated mammalian sperm.

In still another embodiment of the invention a method for affecting the fertility of mammals is provided. The method comprises: applying to a female mammalian reproductive tract a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin, in an amount sufficient to attenuate the desensitization of or to enhance the response of mammalian sperm to odorants.

In another embodiment of the invention a composition for affecting the fertility of female mammals is provided. The composition comprises: a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin, in a pharmaceutically acceptable vehicle suitable for administration to the mammalian female reproductive tract.

The present invention thus provides the art with methods for inhibiting the desensitization of mammals to odorants and for enhancing the perception of odorants. In addition, it provides the art with new methods and compositions for affecting the fertility of mammals.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A through Figure 1D show Nomarski color photomicrographs illustrating the localization of βARK-2 to olfactory cilia. Figure 1A shows staining of olfactory mucosa with affinity purified antibody to βARK-2. Figure 1B shows olfactory tissue which contains respiratory epithelium. Figure 1C is like Figure 1A, but following unilateral bulbectomy. Figure 1D is like Figure 1A, but the sample is primary cultures of olfactory receptor neurons. Figure 1E is like Figure 1A, but using antibody to βARK-1. Figure 1F is like Figure 1B but using antibody to βARK-1. Bar = 50 μm.

Figure 2 shows β-arrestin immunostaining of olfactory epithelium. Figure 2A shows olfactory mucosa stained with affinity-purified antibody to β-arrestin-2. Figure 2B shows β-arrestin-2 immunostaining of respiratory epithelium. Figure 2C is like Figure 2A but after unilateral bulbectomy. Figure 2D and E show β-arrestin-2 immunoreactivity in primary cultures of olfactory receptor neurons. Figure 2F shows β-arrestin-1 immunoreactivity in olfactory epithelium. Figure 2G shows β-arrestin-1 immunoreactivity in respiratory epithelium. Bar = 40 μm.

Figure 3 shows western blot analysis with antibodies to βARK-2 and β-arrestin-2. Bands of relative molecular mass 77 KD (open arrow) and 45 KD

(closed arrow) are visualized respectively in olfactory cilia (CL). Immunoblot analysis failed to detect βARK-1 or β-arrestin-1. Lanes are as follows: 1-βARK-1; 2-βARK-2; 3-β-arrestin-1; 4-β-arrestin-2. The position of molecular weight standards are indicated in kilodaltons.

Figure 4A shows a cross section of rat testes illustrating dense labeling of spermatozoa within the seminiferous tubule by βARK-2 and β-arrestin-2 antibodies. Figure 4B shows βARK-1 immunoreactivity in rat testes. Figure 4C shows a cross section of testes illustrating labeling of spermatozoa with antibodies to β-arrestin-2 within the seminiferous tubule, open arrows indicate labeling of basement membrane which contains specialized smooth muscle. Figure 4D demonstrates β-arrestin-1 immunoreactivity in rat testes. Bar = 50 μm.

Figure 5 shows the effect of neutralizing antibodies on odorant-induced elevation of cAMP levels in isolated rat olfactory cilia. In the absence of odorants (x) there is no significant change in cAMP levels. In the presence of 100 μM citralva, cAMP levels rapidly increase to peak at 50 msec to a value of 47 pmol mg protein (○). In the presence of antibodies to βARK-1 (□) or β-arrestin-1 (△) there is some enhancement of maximal cAMP levels, and loss of decline in cAMP levels at subsequent time points. Preincubation with antibodies to βARK-2 (■) results in a dramatic enhancement of peak cAMP levels, to 220 pmol/mg protein, which is maintained at later time points. In the presence of antibodies to β-arrestin-2 (▲) an increase in peak cAMP levels to 151 pmol/mg protein and loss of desensitization of the response is also seen. When cilia were preincubated with both antibodies to

βARK-2 and β-arrestin-2 (●), there was an additive effect on the enhancement of cAMP levels, reaching a peak of 380 pmol/mg protein which declined only modestly thereafter.

DETAILED DESCRIPTION

It is a discovery of the present invention that an extraordinarily high level of proteins immunoreactive with antibodies specific for β-ARK-2 and βarrestin-2 are found in the dendritic knobs and in the cilia of olfactory receptor neurons in the neuroepithelium, with complete absence of immunoreactivity to antibodies to βARK-1 and β-arrestin-1 in these processes. Additionally, utilizing neutralizing antibodies to the isoforms of βARK-2 and β-arrestin-2 in functional studies, it has now been found that antibodies to βARK-2 and β-arrestin-2 prevent desensitization to odorants, demonstrating that these olfactory proteins serve a specialized role in olfactory desensitization. Whether the isoforms of βARK and β-arrestin localized to the olfactory neuroepithelium represent the same isoforms of βARK-2 and β-arrestin-2 as previously isolated from bovine brain tissue is unclear (Benovic et al., J. Biol. Chem. 266, 14939 (1991); Attramadal et al., J. Biol. Chem. 267, 17882 (1992)). Certainly, the isoforms present in the olfactory epithelium bear strong similarities to βARK-2 and β-arrestin-2, as they react with antibodies which specifically recognize these two isoforms. However, the possibility exists that the βARK and β-arrestin isoforms present in olfactory tissue may indeed be novel members of this groups of proteins. The olfactory isoforms are referred to herein as olfactory ßARK and olfactory ß-arrestin.

The extraordinarily high concentration of βARK-2 and β-arrestin-2 within the olfactory cilia and the ability of neutralizing antibodies to βARK-2 and β-arrestin-2 to elevate the odorant-induced elevation in cAMP levels and to attenuate desensitization (see Examples, below) indicate a role for these proteins in quenching of signal transduction in olfactory signal processing. Such a role is supported by the recent finding that the putative odorant receptors are G-protein coupled, seven transmembrane domain proteins. Several members of this receptor family have been shown by in vitro studies (J.L. Benovic et al., Ann. Rev. Cell Biol., 4, 405 (1988); W.P. Hausdorff et al., FASEB J., 4, 2881 (1990)) to be affected by receptor kinases of the β-ARK family as well as by β-arrestin (M.J. Lohse et al., Science 248, 1547 (1990); J.L. Benovic et al., Proc. Natl. Acad. Sci., USA 84, 8879 (1987)). However, it was not previously known that neutralization of these two proteins would result in attenuation of desensitization and enhancement of odorant perception.

In addition to playing a role in signal transduction in olfactory tissues, the present inventors have found that isoforms of β-ARK-2 and β-arrestin-2 are also present in spermatids. The spermatid isoforms appear to have the same physical characteristics as the olfactory isoforms. The recent finding of members of the odorant receptor family within testes and the selective enrichment of βARK-2 and β-arrestin-2 isoforms within these structures suggest that these particular isoforms of βARK and β-arrestin may be intimately related with the function of this odorant/testis multigene family. A likely role for the odorant receptor family in

sperm is in chemotaxis. Thus inhibition of βARK-2 and/or β-arrestin-2 in sperm will affect fertility.

Compounds which can be used according to the present invention to inhibit olfactory forms of βARK or β-arrestin include polyanions, such as acid mucopolysaccharides, *e.g.* heparin and dextran sulfate, as well as polycations such as polylysine, spermine, and spermidine. Inhibitory compounds also include antibodies which are specific for these olfactory proteins, including anti-βARK-2 and anti-β-arrestin-2. In addition, peptides having the amino acid sequence of domains of the odorant receptor proteins can be used as inhibitors. See L. Buck and R. Axel, Cell 65, 175 (1991). Preferably these will be intracellular domains which are phosphorylated upon binding of the receptor to the odorant. Peptides having the sequences of domains of the β-adrenergic receptor can also be used. These peptides include amino acids 219-243, 56-74, and 57-71 of the β-adrenergic receptor. (Dohlman *et al.*, *Biol. Chem.*, *262*, 14282 (1987)). Such peptides may be cyclized to render them more stable. They may also be attached to other proteins or polymeric substances to provide additional desirable properties, such as to facilitate their uptake by cells or to render them more stable. Compounds can be tested for the ability to inhibit olfactory forms of βARK-2 and/or β-arrestin-2 using a rapid quench technique, as taught in Example 4 below.

Administration of inhibitory compounds to subjects can be accomplished according to any of the known techniques for administering drugs to nasal tissue. These include use of aerosols, nasal sprays or inhalers, and application of topical

solutions or ointments. For administration to the female reproductive tract for affecting fertility, the inhibitors of olfactory forms of βARK-2 and/or β-arrestin-2 can be administered in jellies or cremes, for example, or other vehicles which are conventional for such applications. The vehicle will be pharmaceutically acceptable and preferably will be sterile. Additional ingredients, such as liposomes, might be used to enhance the uptake of the inhibitors by the target cells. The amount of active ingredient in such formulations can be readily determined by routine tests.

According to certain embodiments of the invention, sperm are treated *in vitro* with inhibitors of olfactory forms of βARK-2 and/or β-arrestin-2. Concentrations of inhibitors to be used can be readily determined as those which affect the ability of the sperm to respond to odorants. Alternatively, concentrations can be determined by measuring the inhibition of phosphorylation of the odorant receptor proteins in sperm by the inhibitor compounds. After treatment with the inhibitor, the sperm can be introduced to a female reproductive tract according to known means for artificial insemination.

According to another aspect of the invention, odorant perception is tested in the presence of inhibitors of olfactory forms of βARK-2 and/or β-arrestin-2. Any system for testing odorant perception can be used. One such system is described in U.S. Patent No. 4,521,541. If the administration of the inhibitors allows perception of an odorant, where previously there was none, the amount of the inhibitor which is effective can be noted and can be used as a personal olfaction enhancer for the individual. If administration of inhibitors successfully enhances

olfactory perception, then one has determined the portion of the olfactory pathway which is impaired in the individual being tested. If the administration of inhibitors is not successful for smell enhancement, then one can conclude that the impairment lies elsewhere, *e.g.*, in the brain.

Kits for testing enhancement of odorant perception are also provided by the present invention. These include odorant, inhibitor of olfactory forms of βARK-2 and/or β-arrestin-2, and a means for indicating perception of an odorant. For example, the device which is disclosed in Figures 13, 14, and 15 of U.S. Patent No. 4,521,541 can be used to test perception of an odorant. Additional components of the kit which may be included are instructions for operation as well as means for administering odorants directly to the nasal epithelium, as discussed above.

EXAMPLES

Example 1

This example demonstrates the localization of βARK and β-arrestin isoforms in the olfactory neuroepithelium.

Affinity-purified rabbit antibodies were raised against glutathione-S-transferase fusion proteins containing the carboxyl terminus of rat βARK-1, βARK-2, β-arrestin-1, or β-arrestin-2. Lohse et al. Science 248:1542 (1990); Benovic et al. PNAS 84:8879 (1987); Benovic et al. J. Biol. Chem. 266:14939 (1991); Attramadal J.Biol. Chem. 267 17882 (1992).

Adult male Sprague-Dawley rats were used for the immunohistochemical studies. After administration of pentobarbital (100 mg/kg) anesthesia, rats were perfused transcardially with PBS followed by 4% freshly depolymerized paraformaldehyde in 0.01M phosphate buffer (PB). The olfactory turbinates and bulbs were removed *en bloc* and post-fixed for 1 hr with 4% paraformaldehyde in PBS followed by cryoprotection in 20% (vol) sucrose in PBS. Sections (10 μm) through the olfactory cilia were cut on a cryostat (Microm) and thaw mounted, dried and stored at 70°C. The slide mounted sections were then brought to room temperature. and subsequently permeabilized with 0.2% Triton x-100 in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 1.5% NaCl). This was followed by blocking the sections for 1 hr in 4% normal goat serum (NGS), 0.1% TX-100 in TBS. The sections were then incubated overnight at 4°C in TBS containing 2% NGS, 0.1% TX-100 and affinity purified fusion protein antibodies to βARK (1:5

dilution), βARK-2 (1:50 to 1:500), β-arrestin-1 (1:50 to 1:100) and β-arrestin-2 (1:25 to 1:00). Dilutions varied depending upon batch of affinity purified antibodies. The sections were then stained with an avidin-biotin-peroxidase system (Vector Laboratories) with diaminobenzidine as a chromogen. Blocking experiments were performed by preadsorping the anti-βARK-1, anti-βARK-2, anti-β-arrestin-1 and anti-β-arrestin-2 antibodies with 100 μg of their respective purified fusion protein antigen. Confocal microscopy was performed and sections were labeled with antibodies as described above, except that goat-anti-rabbit antibody conjugated with rhodamine (1:100) (Jackson Immunoresearch Laboratories) was used as the secondary antibody for visualization. Control sections were incubated with pre-immune serums in place of the primary antibodies. After washing, sections were mounted in 10% glycerol in PBS with 1 mg/ml r-phenylenediamine dihydrochloride. The confoncal information was analyzed using a Nikon Opiphot microscope module connected to a laser scanning confocal imaging system (Biorad MRC600). Computer assisted analysis was taken simultaneously for two channels with a 514 nm argon ion laser excitation path between the two filters.

βARK-2 immunoreactivity is evident in the olfactory neuroepithelium (Fig. 1A), whereas βARK-1 immunoreactivity is completely absent (Fig. 1E). These results contrast with our previous studies (Attramadal, supra; Arriza et al., J. Neurosci., 12, 4045 (1992)), in which almost all sites examined thus far contain considerably higher levels of βARK-1 than βARK-2. βARK-2 is localized to the apical surfaces of the olfactory neuroepithelium and appears to be enriched in the

olfactory dendrites (arrows) dendritic knobs (DK), and olfactory cilia (CL). Olfactory receptor neurons (ORN) also occasionally stain lightly. The basal lamina is devoid of staining. Preadsorption with purified βARK-2 fusion protein completely eliminates staining. Localization of βARK-2 to olfactory cilia was confirmed by confocal microscopy (data not shown). βARK-2 is also evident but less prominent, in the outer third of the epithelium, a region consisting of sustentacular cells as well as dendrites of the olfactory receptor neurons. This pattern of immunoreactivity is similar to that seen for the Gs-like protein, G_{olf}, which has been implicated in olfactory signal transduction (D.T. Jones et al., Science 244, 790 (1989)), type III adenylyl cyclase which is highly enriched in olfactory neoronal cilia (H.A. Bakalyar et al., Science 250, 1403 (1989)), and inositol 1,4,5-trisphosphate receptor which has also been implicated in olfactory signal transduction (A.M. Cunningham, D.K. Ryugo, A.H. Sharp, R.R. Reed, G.V. Ronnett and S.H. Snyder, unpublished observations (1992)). Putative odorant receptors (L. Buck et al., Cell 65, 175 (1991)) have also been localized in olfactory neurons and their cilia (A.C. Cunningham et al., Neurosci. Abstr, 17, 75.4 (1991)). In contrast, βARK-2 is completely absent from the region of the adjacent respiratory epithelium (Fig. 1B). Both olfactory neuroepithelium and respiratory epithelium show a complete absence of immunoreactivity to βARK-1 (Fig. 1E & F, respectively).

To confirm the localization of βARK-2 to the distal processes of the olfactory receptor neuron, several additional experiments were performed. The

olfactory receptor neurons of the olfactory epithelium degenerate approximately one week after the olfactory bulb (its target tissue) is removed. (F.L. Margolis et al., Brain Res., 81, 469 (1974)). This technique of neuronal depletion leaves residual olfactory neuroepithelium consisting primarily of sustentacular cells. We have confirmed the presence of βARK-2 in the olfactory receptor neuron dendritic processes and cilia by performing unilateral bulbectomies (Fig. 1C). Following bulbectomies, βARK-2 immunoreactivity is dramatically reduced within the olfactory neuroepithelium. Several dendritic processes (Fig. 1C, open arrows) are residually present and still show immunoreactivity to βARK-2. Note the loss of immunoreactivity in the olfactory cilia and dendritic knobs.

Bulbectomies were performed unilaterally allowing comparison of normal and denervated sides. Adult male Sprague-Dawley rats were anesthetized with chloral hydrate (0.5g/kg, IP) and placed in a stereotaxic head holder. The olfactory bulb was identified on the right side of all animals and subsequently aspirated with a glass pipette. Following bulbectomy, the surgical site was packed with gel foam and the skin was sutured with surgical staples. Animals were allowed to recover and were subsequently prepared for immunohistochemistry as described above.

Additionally, primary cultures of neonatal rat olfactory receptor neurons were prepared and examined for βARK-1 and βARK-2 immunoreactivity (Fig. 1D). Whereas βARK-1 immunoreactivity is absent, βARK-2 immunoreactivity is seen throughout the cell body (arrow) and processes, as well as in the region of the putative dendritic knob (open arrow). Primary cultures of olfactory receptor

neurons were prepared on two-chamber Labtek tissue culture slides as previously described [G.V. Ronnett, L.D. Hester and S.H. Snyder, J. Neurosci., 11, 1243 (1991)]. After 5 to 7 days in culture, the slides were rinsed 3 times with phosphate buffered saline (PBS, pH 7.4) and placed in methanol at -20°C for 15 to 20 min. Immunohistochemistry was performed as described above.

Since additional quenching of the activity of G protein coupled receptors requires the presence of an arrestin-like protein (J.L. Benovic et al., Ann. Rev. Cell Biol., 4, 405 (1988); W.P. Hausdorff et al., FASEB J., 4, 2881 (1990), Lohse et al., Science 248, 1547 (1990); Benovic et al., Proc. Natl. Acad. Sci., USA 84, 8879 (1987)) we also examined the distribution of β-arrestin-1 and β-arrestin-2 within the olfactory neuroepithelium. β-arrestin-2 is enriched within the apical dendritic knobs (DK) (open arrows) of olfactory receptor neurons as well as the neurons themselves (ORN) which express a moderate amount of immunoreactivity (Fig. 2A). The basal lamina is devoid of staining. Preadsorption with purified fusion protein completely eliminates staining. Interestingly, the distribution of β-arrestin-2 appears slightly different than that of βARK-2 at the light microscopic level. Whereas β-arrestin-2 is most concentrated in dendritic knobs, βARK-2 is most prominent in the dendritic knobs and ciliary layer. This is consistent with previous localization studies (Attramadal et al., supra; Arriza et al., supra), where β-arrestin and βARK have been shown to localize within the same set of neurons although in different subcellular compartments. Confocal microscopy was performed and the presence of β-arrestin-2 in olfactory dendritic knobs was

confirmed (data not shown). In contrast, immunoreactivity for β-arrestin-1 is completely absent (Fig. 2E).

Following bulbectomy, β-arrestin-2 immunoreactivity is markedly attenuated both within the apical dendritic knobs and olfactory receptor neurons thus indicating a specific localization to olfactory receptor neuronal process. (Fig. 2C.) Note the staining occasional ORNs (arrows) which are regenerating and residual dendritic knobs (open arrows).

Primary cultures of olfactory receptor neurons were examined for β-arrestin-immunoreactivity (Fig. 2D). Open arrows indicate putative dendritic knob and arrows indicate cell bodies. Whereas β-arrestin-1 is absent from cultured cells, β-arrestin-2 is seen in the cell processes in a distribution similar to that seen for βARK-2. Within respiratory epithelium β-arrestin-2 was completely absent; however, interestingly β-arrestin-1 is present.

Example 2

This example demonstrates that the antisera used for immunohistochemistry and immunocytochemistry recognize βARK-1 and βARK-2, β-arrestin-1 and β-arrestin-2.

We conducted immunoblot analysis (Fig. 3). Cytosolic extracts were prepared from rat olfactory cilia, olfactory turbinates, primary olfactory cultures and testes. The olfactory tissues were homogenized with a Brinkman Polytron in 2 ml of 50 mM Tris pH 7.5, 5 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 5 μg/ml pepstatin, and 10 μg/ml benzamidine and centrifuged at 300,000

x g for 20 min at 4°C and the pellets were discarded. Protein samples (100-150 μg/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels for olfactory tissues and the separated proteins were transferred to nitrocellulose. All samples for the anti-β-arrestin antibodies were treated with iodoacetamide added to a final concentration of 0.5 M for 30 min in the dark prior to SDS-PAGE. Blots were then blocked with 3% BSA in TBS and then incubated with affinity-purified antibodies at dilutions of 1:30 to 1:2000. Dilutions varied depending upon the batch of the primary antibody. Blots were developed using a goat anti-rabbit alkaline phosphatase kit (BioRad) according to the manufacturer's protocol except for the use of 3% BSA instead of gelatin in all antibody buffers.

In olfactory cilia βARK-2 antiserum interacts with a single band of relative molecular mass 77 kD (open arrow) (J.L. Arriza et al., J. Neurosci., 12, 4045 (1992)) and β-arrestin-2 interacts with single band of relative molecular mass 45 kD (closed arrows) (Attramadal, supra), the same as expressed βARK-2 and β-arrestin-2 in transfected COS cells respectively (Fig. 3), thus confirming the presence of both βARK-2 and β-arrestin-2 isoforms in olfactory cilia. Therefore, although the most prominent staining of β-arrestin-2 is seen in the dendritic knobs, significant amounts must also be present in the cilia preparations as β-arrestin-2 immunoreactivity is detected in immunoblot analysis. Immunoblot analysis failed to detect βARK-1 or β-arrestin-1. Primary olfactory receptor neurons in culture gave a similar but less prominent staining pattern (data not shown). The exclusive presence of βARK-2

versus βARK-1, as well as the exclusive presence of β-arrestin-2 versus β-arrestin-1, is in contrast to essentially all other tissue and brain regions previously examined.

Example 3

This example demonstrates the presence of βARK-2 and β-arrestin-2 isoforms in spermatozoa of testes.

Recently a large G protein-coupled receptor gene family has been identified from an olfactory cDNA library (L. Buck et al., Cell 65, 175 (1991)). Interestingly, some of these gene products were also identified within germ cells (M. Parmentier et al., Nature, 355 453 (1992)). To further investigate the potential role of βARK and β-arrestin in signal desensitization as related to this gene family, we have examined the distribution of these proteins with antibodies to βARK and β-arrestin in rat testis. Rat tissue was prepared and immunohistochemistry was performed essentially as described above for olfactory tissues. Both βARK-2 and β-arrestin-2 are enriched within mature spermatids (Fig. 5 A&C), with complete absence of βARK-1 (Fig. 5B) and β-arrestin-1 (Fig. 5D).

The testes was homogenized with a Brinkman Polytron in 10 vols/wet weight tissue in 50 mM Tris pH 7.4 containing 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 4.8 μg/ml antipain, 9.6 μg/ml leupeptin A. Testicular homogenates were centrifuged for 30 min at 45,000 x g and the pellets were discarded. The supernatants were assayed for protein using the coomassie blue assay from Pierce or by the method of Bradford [M.M. Bradford. Anal. Biochem., 72:248 (1976)].

Similar to olfactory cilia, immunoblot analysis of rat testis shows that βARK-2 antiserum labels a single band of 77 kD and β-arrestin-2 antiserum labels a single band of 45 kD, whereas both βARK-1 and β-arrestin-1 are absent (data not shown).

Example 4

This example demonstrates that antibodies to βARK-2 and β-arrestin-2 enhance the response of olfactory cilia to odorants and attenuate their desensitization.

Odorants induce elevations of cAMP levels (Pace et al., *supra*; Sklar et al., *supra*; Breer et al., *supra*; Ronnett et al., *supra*;) via the activation of adenylyl cyclase and this increase in cAMP initiates the electrophysiologic response by gating a nonspecific cation channel in the sensory cilia (R.S. Dhallan et al., Nature 347, 184 (1990); J. Ludwig et al., FEBS Lett. 270, 24 (1990); S. Firestein et al., J. Neurosci., 11, 3565 (1991); T. Nakamura et al., Nature 325, 442 (1987)). In response to odorants, adenylyl cyclase is rapidly and only transiently activated, thereby suggesting that active desensitization is involved in the uncoupling of the receptor-G-protein-adenylyl cyclase pathway. The immunocytochemical localization of βARK-2 and β-arrestin-2 to olfactory cilia suggests that these proteins may function in homologous desensitization in response to odorants within the olfactory system. To investigate this possibility, we conducted functional studies, utilizing a rapid quench system (I. Boekhoff et al., EMBO J., 9, 2453 (1990)), to monitor cAMP levels in response to odorants in the presence or absence of neutralizing antibodies to the isoforms of βARK and β-arrestin.

Rat olfactory cilia were isolated by calcium shock (P.B. Sklar et al., J.Biol. Chem. 261 15538 (1986)). A rapid quench device, designed by Update Instrument, Inc., Madison, WI, was used to measure cAMP levels at the millisecond range using three syringes. The first syringe contained cilia suspended in buffer consisting of 10 mM Tris-HCl, 3 mM $MgCl_2$, and 2 mM EDTA, pH 8.0. The second syringe contained the reaction mix consisting of 200 mM NaCl, 10 mM EGTA, 40 mM MOPS, 2.5 mM $MgCl_2$, 1.0 mM DTT, 1.0 mM Na-ATP, 1.0 μM GTP, 0.05% Na-cholate, and citralva at appropriate concentrations, pH 7.4. The free Ca^{2+} in this solution was 45 nM as measured by a Ca^{2+} electrode (Orion). The third syringe contained 13.5% trichloroacetic acid. A time course with intervals ranging from 20 ms to 500 ms was used and the quenched samples were collected on ice and spun 10 min in a microfuge at 4°C. The supernatant was collected and cAMP was assayed using the Amersham [^{125}I] cAMP assay system. Protein concentration in the first syringe averaged 150 μg/ml cilia buffer, as determined by the method of Lowry [O.H. Lowry et al., J. Biol. Chem., 2, 265 (1951)].

Cyclic AMP levels were rapidly elevated from their basal level of 11.3 pmol/mg protein to 47 mol/mg protein at 50 msec by citralva, and the levels declined rapidly thereafter, to 20 pmol/mg protein at 500 msec, suggesting active desensitization (Fig. 4). In the absence of stimulation by citralva, antibodies to βARK and β-arrestin did not alter levels of cAMP. Thirty minute pretreatment of cilia with anti-β-arrestin-2 antibodies (25 μg/ml) results in an elevation of peak cAMP levels in the presence of 100 μm citalva to 151 pmol/mg protein with a

decline at 500 msec to 130 pmol/mg protein. Preincubation with anti-βARK-2 antibodies (25 μg/ml results in an even higher peak cAMP response due to citralva, to 220 pmol/mg protein declining to 187 pmol/mg protein at 500 msec. Preincubation with a combination of anti-βARK-2 and anti-β-arrestin-2 antibodies resulted in additive effects, with a peak cAMP level of 380 pmol/mg protein which subsequently declined to 262 pmol/mg protein at 500 msec. Presumably the slower decline of cAMP levels in the presence of anti-βARK-2 and/or anti-β-arrestin-2 antibodies reflects blockade of desensitization.

Preincubation of anti-βARK-1 antibody at 25 μg/ml results in some elevation of the peak cAMP levels to 58 pmol/mg protein, which did not decline at subsequent time points. Similar results were obtained by preincubating the cilia with anti-β-arrestin-1 antibody (25 μg/ml). At the concentration of neutralizing antibody utilized in these experiments, both anti-βARK-1 and anti-β-arrestin-1 probably cross react with the endogenous isoforms of βARK and β-arrestin within olfactory cilia (H. Attramadal et al., J. Biol. Chem., 267:17882 (1992), J.L. Arriza et al., J. Neurosci., 12, 4045 (1992)), thereby accounting for the modest effect on cAMP levels. In contrast, preincubation of cilia with rabbit IgG at comparable concentrations did not affect cAMP levels in response to citralva.

CLAIMS

1. A method for inhibiting the desensitization to odorants or for enhancing the perception of odorants in a subject comprising:

administering a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin, to olfactory epithelium of the subject in an amount sufficient to inhibit desensitization or to enhance the perception of an odorant.

2. The method of claim 1 wherein the molecule is heparin.

3. The method of claim 1 wherein the molecule is an antibody which specifically binds to olfactory βARK or olfactory β-arrestin.

4. The method of claim 1 wherein the molecule is administered as an aerosol.

5. The method of claim 1 wherein the molecule is administered in a topical solution.

6. The method of claim 3 wherein said antibody is anti-βARK-2.

7. The method of claim 3 wherein said antibody is anti-β-arrestin-2.

8. A kit for diagnosing a cause of anosmia, comprising:

one or more odorants;

a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin;

a means for indicating perception of an odorant.

9. The kit of claim 8 wherein said molecule is heparin.

10. The kit of claim 8 wherein said molecule is an antibody which specifically binds to olfactory βARK or olfactory β-arrestin.

11. The kit of claim 10 wherein said antibody is anti-βARK-2.

12. The kit of claim 10 wherein said antibody is anti-β-arrestin-2.

13. A process for testing odorant perception in a subject who cannot smell an odorant, comprising the steps of:

exposing the subject to an odorant at a concentration at which the subject cannot perceive the odorant;

administering to the subject a molecule which inhibits an olfactory protein selected from the group consisting of βARK and β-arrestin;

determining whether administration of said antibody allows the subject to perceive the odorant.

14. The process of claim 13 wherein a variety of concentrations of the molecule are administered, and the minimum concentration required to allow the subject to perceive the odorant is determined.

15. The process of claim 13 wherein the molecule is heparin.

16. The process of claim 13 wherein the molecule is an antibody which specifically binds to olfactory βARK or olfactory β-arrestin.

17. The process of claim 16 wherein said antibody is anti-βARK-2.

18. The process of claim 16 wherein said antibody is anti-β-arrestin-2.

19. A method for affecting fertility of mammals, comprising:

applying to mammalian sperm a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin in an amount sufficient to attenuate the desensitization or enhance the response of said sperm to odorants; and

artificially inseminating a female mammal with said treated mammalian sperm.

20. The method of claim 19 wherein the molecule is heparin.

21. The method of claim 19 wherein the molecule is an antibody which specifically binds to olfactory βARK or olfactory β-arrestin.

22. The method of claim 21 wherein said antibody is anti-βARK-2.

23. The method of claim 21 wherein said antibody is anti-β-arrestin-2.

24. A method for affecting the fertility of mammals, comprising:

applying to a female mammalian reproductive tract a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin, in an amount sufficient to attenuate the desensitization or enhance the response of mammalian sperm to odorants.

25. The method of claim 24 wherein the molecule is heparin.

26. The method of claim 24 wherein the molecule is an antibody which specifically binds the olfactory βARK or olfactory β-arrestin.

27. The method of claim 26 wherein said antibody is anti-βARK-2.

28. The method of claim 26 wherein said antibody is anti-β-arrestin-2.

29. A composition for affecting the fertility of mammals, comprising:

a molecule which inhibits an olfactory protein selected from the group consisting of: β-adrenergic receptor kinase (βARK) and β-arrestin, in a pharmaceutically acceptable vehicle suitable for administration to the mammalian female reproductive tract.

30. The composition of claim 29 wherein the molecule is heparin.

31. The composition of claim 29 wherein the molecule is an antibody which specifically binds to olfactory βARK or olfactory β-arrestin.

32. The composition of claim 31 wherein said antibody is anti-βARK-2.

33. The composition of claim 31 wherein said antibody is anti-β-arrestin-2.

FIG. 1A

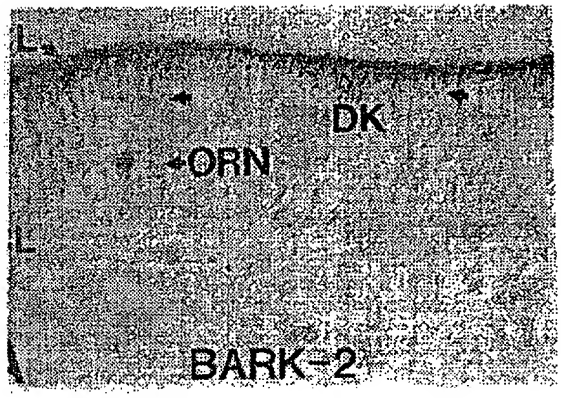


FIG. 1B

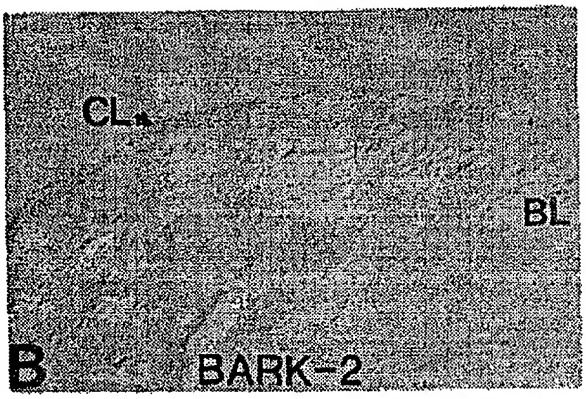


FIG. 1C

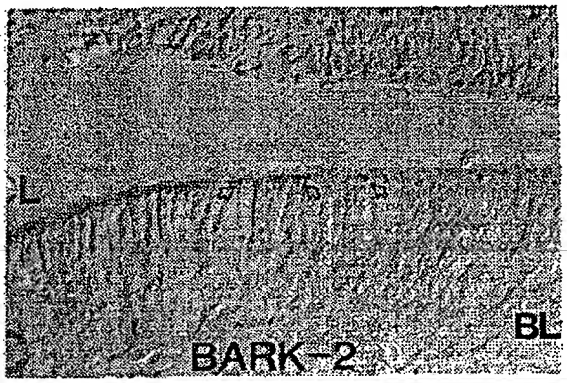


FIG. 1D

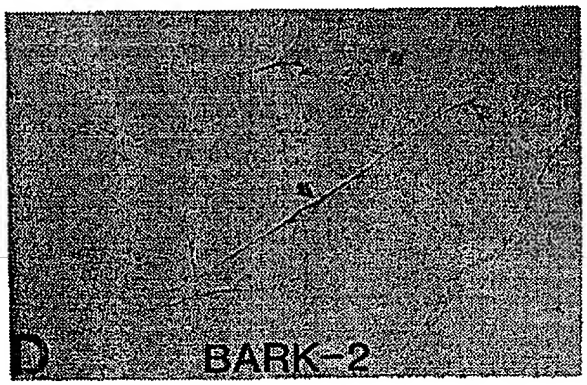


FIG. 1E

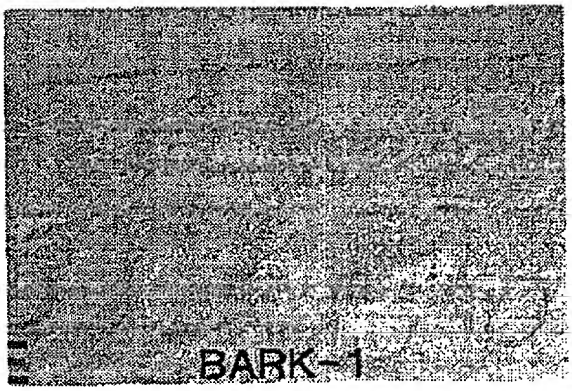


FIG. 1F

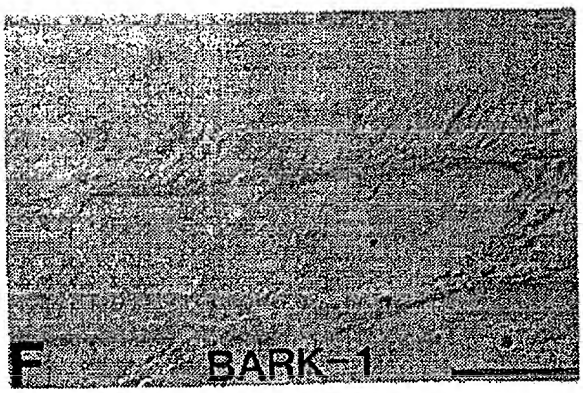


FIG. 2A

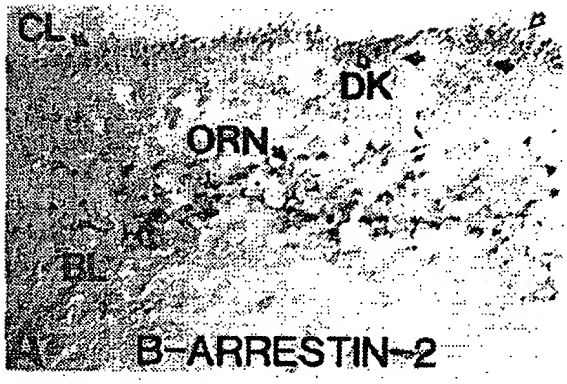


FIG. 2B

FIG. 2C

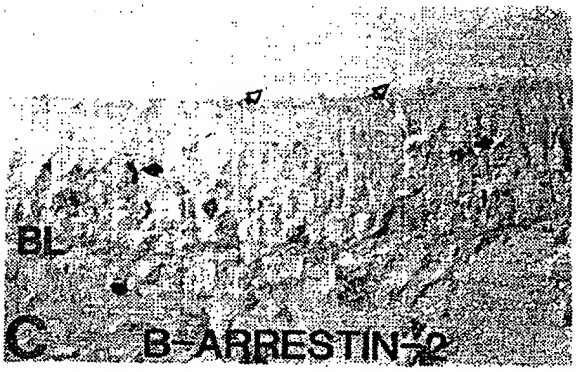


FIG. 2D

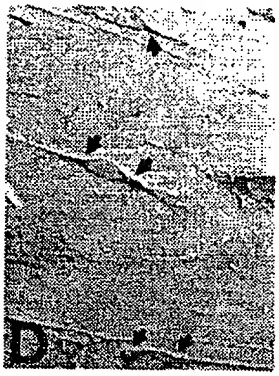


FIG. 2E

FIG. 2F

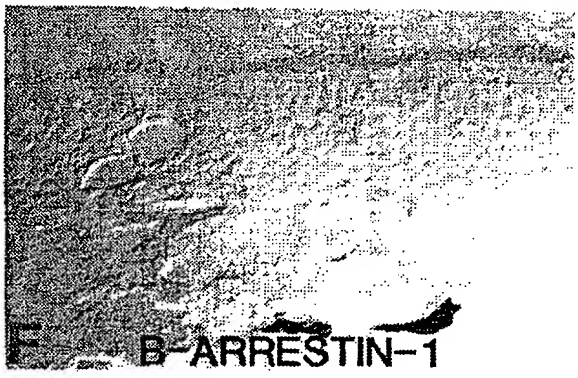


FIG. 2G

FIG. 3

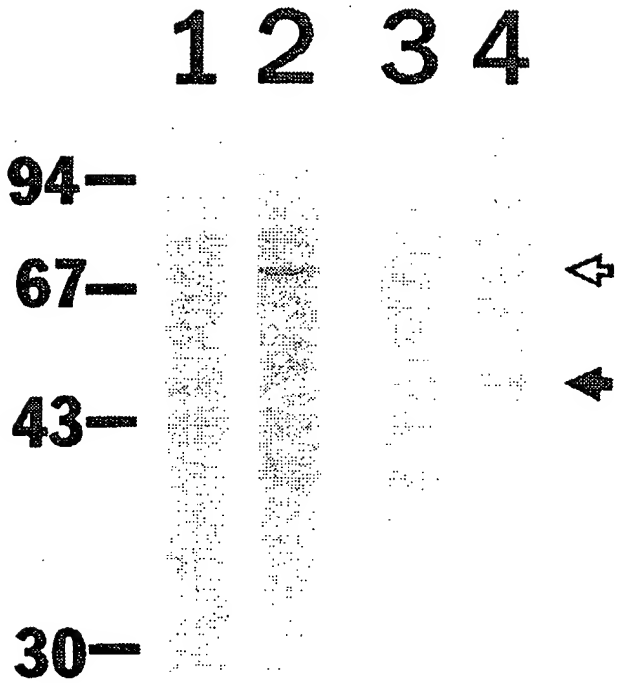


FIG. 4A

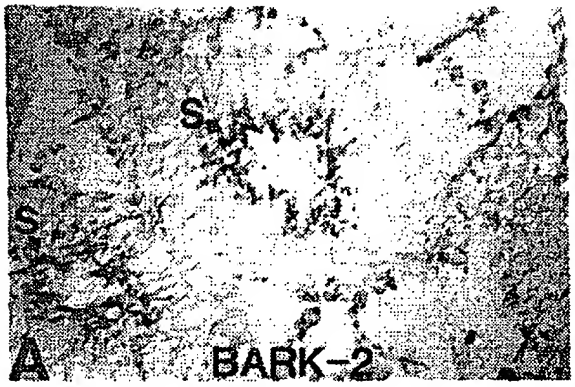
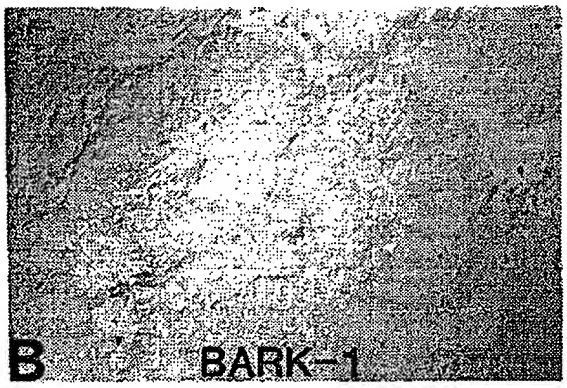


FIG. 4B



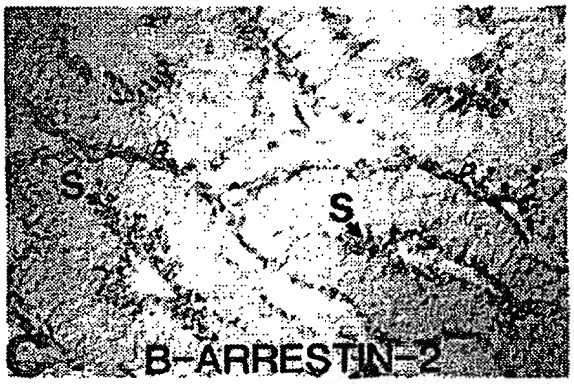


FIG. 4C

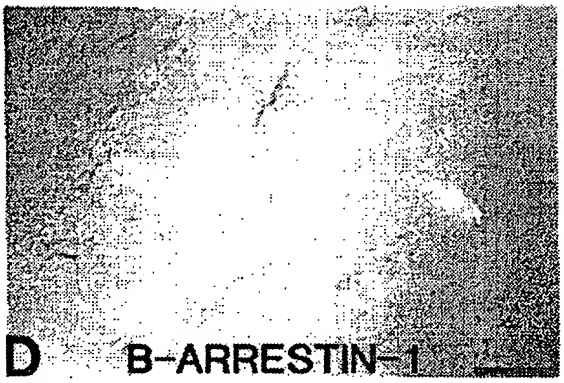
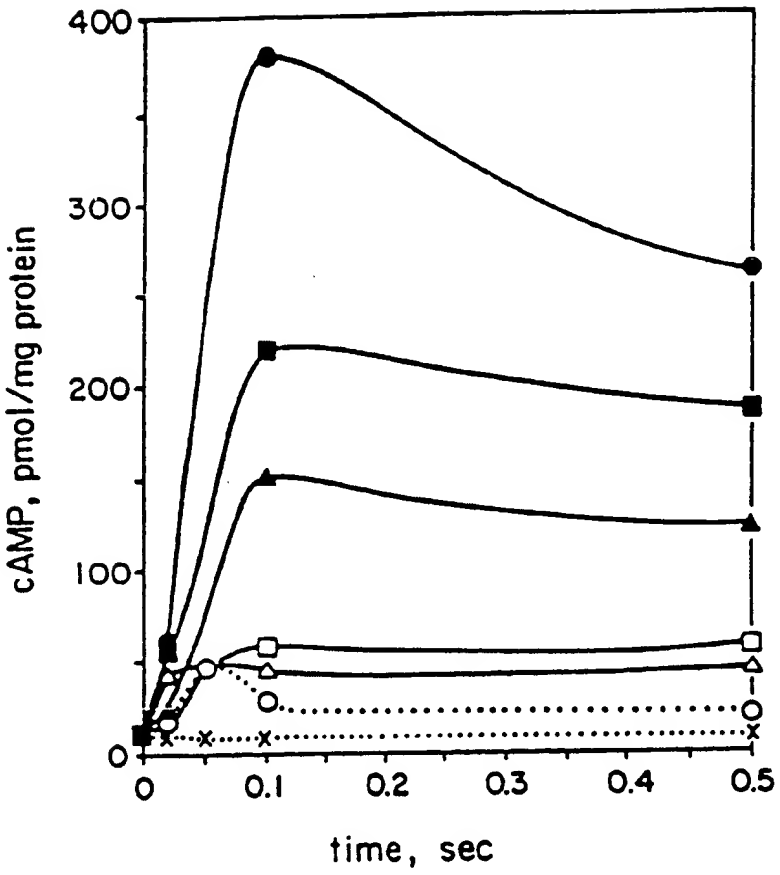


FIG. 4D

FIG. 5



INTERNATIONAL SEARCH REPORT

Inte: nal Application No
PCT/US 94/00516

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K31/00 A61K31/725 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCIENCE vol. 259, no. 5096 , 1993 pages 825 - 829 T.M. DAWSON ET AL 'B-adrenergic receptor kinase-2 and B-arrestin-2- as mediators of odorant-induced desensitization.' see the whole document ---	1,3,6,7
A	J.BIOL. CHEM. vol. 267, no. 25 , 1992 pages 17882 - 17890 H. ATTRAMADAL ET AL 'B-Arrestin-2, a novel member of the arrestin/B-arrestin gene family.' --- -/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 1994	29. 04. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Klaver, T

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/00516

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE vol. 248, no. 4962 , 1990 pages 1547 - 1550 M.J. LOHSE ET AL 'B-Arrestin: a protein that regulates B-adrenergic receptor function.' cited in the application	

6. The conjugate of claim 1 wherein the ligand is derived from anandamide and specifically binds to cannabinoid receptors, and the fluorescent label is derived from dansyl chloride.

7. The conjugate of claim 1 wherein the ligand is derived from dichlorokynurenic acid and specifically binds to glycine receptors, and the fluorescent label is derived from nitrobenz-2-oxa-1,3-diazol-4-yl.

8. The conjugate of claim 1 wherein the ligand is derived from MK 801 and specifically binds to N-methyl-D-aspartate receptors, and the fluorescent label is derived from dansyl chloride.

9. The conjugate of claim 1 wherein the ligand is derived from glibenclamide and specifically binds to potassium channel receptors, and the fluorescent label is derived from nitrobenz-2-oxa-1,3-diazol-4-yl.

10. The conjugate of claim 1 wherein the ligand is derived from procainamide and specifically binds to sodium channel receptors, and the fluorescent label is derived from dansyl chloride.

* * * * *